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Date: 23 June 1997

Kilburn & Strode

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INVENTION

The present invention relates to nucleic acid sequencing.

According to one aspect of the present invention there is provided a method for sequencing nucleic acid molecules, comprising the steps of:

a) providing at a first location a plurality of single stranded nucleic acid molecules that have the same sequences as one another and that are hybridised to primers in a manner to allow primer extension in the presence of nucleotides and a nucleic acid polymerase;

b) providing at a second location, which is different from the first location, a plurality of single stranded nucleic acid molecules that have the same sequences as one another, but that have different sequences from the sequences of the single stranded nucleic acid molecules at the first location, and that are also hybridised to primers in a manner to allow primer extension in the presence of nucleotides and a nucleic acid polymerase;

c) providing each location with a nucleic acid polymerase and a given labelled nucleotide under conditions that allow extension of the primers if a complementary base or if a plurality of such bases is present at the appropriate position in the single stranded nucleic acid molecules;

d) detecting whether or not said labelled nucleotide has been used for primer extension at each location by determining whether or not the label

present on said nucleotide has been incorporated into extended primers;

e) repeating steps c) and d) one or more times so that extended primers comprising a plurality of labels are provided.

This aspect of the present invention allows labelled nucleotides to be incorporated in a stepwise manner in a nucleic acid molecule via primer extension. The presence of one labelled nucleotide in the molecule does not prevent other labelled nucleotides being detected (even if adjacent labelled nucleotides are the same). Thus, unlike many prior art methods, there is no need to remove a label from a polynucleotide chain before a further labelled nucleotide is added (although in some embodiments it may be desired to remove labels periodically). A plurality of labels can therefore be incorporated into a nucleic acid molecule via primer extension and can be detected *in situ*.

The method is also advantageous over prior art methods that rely upon the use of large numbers of different oligonucleotide tags in determining nucleotide sequences such as the methods disclosed by Lynx Therapeutics Inc. in WO96/12014 and WO96/12039. These methods are generally time-consuming and require specific tags to be obtained/synthesised. They may also use restriction enzyme digestion or other means to remove tags or parts thereof following a detection step. The present invention does not suffer from these drawbacks.

A further advantage of the present invention is that it allows high sensitivity because labels are directly incorporated into a plurality of identical nucleic acid

molecules that are present at a single location. This allows a sharp signal to be produced and detected *in situ*.

The single stranded molecules that are hybridised to primers in steps a) and b) above are referred to herein as "templates". These may be DNA or RNA molecules. They may comprise naturally occurring and/or non naturally occurring nucleotides.

Preferably the template molecules are immobilised on a surface - i.e. they are maintained in position on a surface by covalent or non-covalent interactions for sufficient time to allow the present invention to be performed. They may be directly or indirectly bound to the surface.

In this aspect of the present invention, each location comprises a plurality of primers hybridised to nucleic acid molecules used as templates in sequencing. (No more than one primer will normally be annealed to each nucleic acid molecule acting as a template molecule. In applications when a given type of template molecule is to be annealed to different primers, sequencing can be performed at different locations.) By providing different template molecules at different locations, the present invention allows the sequencing of different nucleic acid molecules to occur in parallel.

Sequencing may be full or may be partial. The sequence of a complementary strand to the template or of a part thereof can be obtained initially. However this sequence can be converted (using base-pairing rules) to provide the sequence of the template or of a part thereof. This conversion can be done via a

computer or via a person. It can be done after each step of primer extension or at a later stage.

Desirably, the method discussed above includes a step of removing labelled nucleotides from the first and second locations if they are not incorporated by primer extension (e.g. via a washing step, as will be described later). By immobilising templates, labelled nucleotides can be removed from the locations of the templates whilst the templates are maintained in position.

Any suitable surface may be provided for immobilising nucleic acid molecules. The term "support" is used herein to describe a material possessing such a surface. The support is preferably a solid material or a semi-solid material (e.g. a gel or a lipidic monolayer or bilayer).

Various protocols are known for binding nucleic acids to a surface. For example they can be covalently bound to the surface of a glass or polymer support (see e.g., Joos, B., Kuster, H., and Cone, R. (1997), entitled "Covalent attachment of hybridizable oligonucleotides to glass supports", *Analytical Biochemistry* 247, 96-101; Oroskar AA. Rasmussen SE. Rasmussen HN. Rasmussen SR. Sullivan BM. Johansson A. (1996), entitled "Detection of immobilized amplicons by Elisa-like techniques", Clinical Chemistry. 42(9):1547-1555; Khandjian EW, entitled "UV crosslinking of RNA to nylon membrane enhances hybridization signals", Molecular Biology Reports. 11(2):107-15, 1986.)

Nucleic acid molecules may also be immobilised to a support via non-covalent binding. The non-covalent binding may be achieved, for example, by interaction between biotinylated nucleic acids and a (strept)avidin coated surface or by the anchoring of an hydrophobic chain into a lipidic monolayer or bilayer.

Indeed any interaction between a surface and a nucleic acid molecule which allows the various steps of the method to be performed may be utilised for binding. For example template molecules may be bound to a surface merely by base pairing with primers already bound to a surface.

In preferred embodiments, a single surface is provided on which template molecules are immobilised. In less preferred embodiments, discrete units each having their own surface may be provided. For example, spheroids may be provided (e.g. glass beads/beads formed of another polymeric material, etc.). Alternatively other three-dimensional units may be provided.

The discrete units may possess magnetic properties or other properties to facilitate separation of the units from other components (e.g. during washing steps). (Magnetic beads can be obtained from Dynabeads™ M-280, Dynal A.S. Oslo, Norway. They can be used as described by Hultman, T., Bergh, S., Moks, T., Uhlen, M. Bidirectional solid-phase sequencing of in vitro-amplified plasmid DNA. BioTechniques 10:84-93, 1991.)

Different units may even have different properties in order to facilitate separation into different categories of unit (e.g. primers of one type may be bound to units having a given property and primers of another type may be

bound to units having a different property or at least not having the given property).

Desirably, a planar surface is provided (e.g. by a membrane or by a glass slide).

It is preferred to provide high densities of immobilised template molecules and/or of primers. Desirably high densities are provided in small areas. This can be achieved using robotic systems. Such systems may rely on piezoelectric delivery systems or other delivery systems in order to deliver small quantities (e.g. nanolitres or even picolitres) of material to small areas. The small areas may be in the form of arrays (that may be regular or irregular). By controlling the operation of a delivery device different types of array may be provided.

In less preferred embodiments arraying can be performed after binding of template and/or primer molecules to a surface. In such a case the molecules can be immobilised on discrete units of support (*e.g.* as discussed above), which are subsequently arrayed. Each unit may therefore have a surface on which immobilised nucleic acids are present.

Desirably, a large number of copies of the same initial template molecule is provided at a given location. For example, over 10,000 such molecules may be provided.

A location may be in the form of a single distinct area (that is preferably planar). Preferably the areas are from 100 nm to 2 cm, more preferably from 500 nm to 5 mm in length, when measured across the largest dimension. In the

case of areas defined by a generally circular perimeter this measurement will be the diameter of the circle.

Less preferably, a location may comprise the contents of a container or a part thereof (*e.g.* template-bound beads in a tube or in a microplate well).

A plurality (two or more) locations will be generally provided having one or more of the characteristics discussed in the foregoing paragraphs.

Where a large number of template molecules are provided at the locations, this can enhance the reliability of sequence data provided. This is because the probability of a false reading being provided due to the presence of contaminants can be minimised.

In less preferred embodiments, the sequencing may be performed on a small number of template molecules provided that sensitive detection techniques are used. (Sensitive detection techniques allowing for detection of single molecules can, for example, utilize:

a) A Cooled Charge Coupled Device (CCD) camera (e.g. Princeton Instruments, with long integration time),

b) Confocal microscopy (e.g. Carl Zeiss Jena, also with long integration),

c) SNOM imaging (Scanning near-field optical microscopy). Jett, J.H., Keller, R.A., Martin, J.C., Marrone, B.L., Moyzis, R.K., Ratliff, R.L., Seitzinger, N.K., Brooks Shera, E., Stewart, C.C. (1989),

or

d) High-speed DNA sequencing: an approach based upon fluorescence detection of single molecules. Journal of Biomolecular Structure & Dynamics 7: 301-309)

Template molecules provided at different locations may be nucleic acid molecules from different sources. These may or may not have one or more identical/homologous sequences along their lengths. For example, different template molecules may have identical/homologous sequences over all/part of their lengths when similar samples from different organisms are provided (e.g. similar genetic regions from related organisms). Alternatively, different samples from the same organism (e.g. samples from different types of cell, tissue or organ) may be provided.

One of the main advantages of the present invention is that sequences, whether full or partial, can be read simultaneously and efficiently from a plurality of different locations. (The present invention is not limited to being applied at only the first and second locations discussed previously.) For example, over 10, over 100, over 1000, or even over 1,000,000 different locations may be provided. Thus many different sequences can be determined in a relatively short time period.

If desired, each type of template molecule can be provided at a plurality of different locations (preferably in amplified form at each location), allowing for redundancy of the sequence data generated. This redundancy allows controls to be provided to provide checks on the accuracy of sequence data generated.

With regard to primers suitable for use in the present invention, oligonucleotides are preferred. These are nucleic acid molecules typically 6 to 60, e.g. 15 to 25 nucleotides long. They may comprise naturally and/or non naturally occurring nucleotides. Analogue molecules such as longer nucleic acid fragments may alternatively be used as primers, if desired.

Primer annealing (hybridisation) to template molecules is preferably performed by heat denaturation followed by slow cooling. Preferred annealing conditions (temperature and buffer composition) prevent non-specific hybridisation. Primers which remained in solution or which did not anneal specifically to the template are preferably removed after annealing.

Stringent annealing conditions can be used which prevent non specific primer hybridisation. These conditions would typically be annealing temperatures close to a primer's Tm (melting temperature) at a given salt concentration (e.g. 50 nM primer in 200 mM NaCl buffer at 55°C for a 20-mer 50% GC oligonucleotide). (Stringent conditions for a given system can be determined by a skilled person. They will depend on the base composition, GC content, the length of the primer used and the salt concentration. For a 20 nucleotide oligo of 50% GC, calculated average annealing temperature is 55-60°C, but in practice may vary between 35

to 70°C).

Primers may be immobilised by being directly linked to a surface by the techniques discussed *supra* for template molecules. Alternatively, they may be indirectly linked to the surface, e.g. by virtue of being annealed to a template molecule that is itself bound to the surface.

In any event the template molecule will comprise a portion that hybridises with the primer (preferably under "stringent" conditions). This portion can be added to a given molecule (even if of totally/partially unknown sequence) using techniques known to those skilled in the art to provide a template. For example it can be synthesised artificially and can be linked via a ligase to the molecule of totally/partially unknown sequence. This can result in a single or double stranded molecule. If a double stranded molecule is produced, heating can be used to separate annealed strands to provide a single stranded template.

Once a template is provided annealed to a primer, primer extension can be performed. RNA or DNA polymerases can be used. DNA polymerases are however the enzymes of choice for preferred embodiments. Several of these are commercially available. Polymerases which lack 3' → 5' exonuclease activity can be used, such as T7 DNA polymerase or the small (Klenow) fragment of DNA polymerase I may be used [*e.g.* the modified T7 DNA polymerase Sequenase™ 2.0 (Amersham) or Klenow fragment (3'→5' exo⁻, New England Biolabs)]. However it is not essential to use such polymerases. Indeed, where it is desired that the polymerases have proof-reading activity polymerases lacking 3' → 5' exonuclease activity would not be used.

Certain applications may require the use of thermostable polymerases such as ThermoSequenase™ (Amersham) or Taquenase™ (ScienTech, St Louis, MO).

Any nucleotides may be used for primer extension reactions (whether naturally occurring or non-naturally occurring). Preferred nucleotides are deoxyribonucleotides dATP, dTTP, dGTP and dCTP or ribonucleotides ATP, UTP, GTP and CTP; at least some of which are provided in labelled form.

The use of labelled nucleotides during primer extension facilitates detection. (The term "label" is used in its broad sense to indicate any moiety that can be identified using an appropriate detection system. Preferably the label is not present in naturally occurring nucleotides.)

Ideally, labels are non-radioactive, such as fluorophores which allow efficient detection of primer extension. In some applications, considering that a large number of copies of each template molecules can be immobilised at each location, one can envisage the use of a combination of labelled and non-labelled nucleotides. In this case, even if a small proportion of the incorporated nucleotides are fluorescence labelled, the number of fluorophores at each location can be sufficient to be detected by a detection device, whilst reducing the possibility of contiguous incorporation of labelled bases which, for example, may give rise to quenching effects between fluorophores.

Contiguous incorporation of labelled bases may be preferred in other applications.

In a preferred embodiment, only one type of label is present on four types of nucleotides to be used in extending a primer. Each nucleotide incorporation can therefore provide a cumulative increase of the same signal (e.g. of a signal measured at a particular wavelength).

After several steps of sequencing during which a plurality of labelled nucleotides have been incorporated into a nucleic acid strand by primer extension, it may be desired to reduce a signal to an initial level or at least to some extent. This can be achieved by removing one or more labels from already incorporated nucleotides (*e.g.* by laser bleaching of the fluorophores).

Alternatively, polymerisation steps may be proceeded with using another type of label from that used initially (*e.g.* switching from fluorescein to rhodamine).

In less preferred embodiments, different labels may be used for each type of nucleotide. For example, different fluorophores may be used.

In other less preferred embodiments, the primer itself and its extension product may be removed and replaced with another primer. If required, several steps of sequential label-free nucleotide additions may be performed before actual sequencing in the presence of labelled nucleotides is resumed.

A washing step is preferably incorporated after each primer extension step in order to remove unincorporated nucleotides which may interfere with subsequent steps. The preferred washing solution should be compatible with

polymerase activity and have a salt concentration which is high enough not to interfere with the annealing of primer molecules and templates.

In less preferred embodiments, the washing solution may not be suitable for polymerase activity. Here the washing solution would need to be removed before further polymerisation occurred.

Various detection systems can be used to detect labels (although in certain embodiments detection may be possible simply by eye, so that no detection system is needed). A preferred detection system for fluorescent labels is a Charge-Coupled-Device (CCD) camera, possibly coupled to a magnifying device. Any other device allowing detection and, preferably, also quantification of fluorescence on a surface may be used. Devices such as fluorescent imagers or confocal microscopes may be chosen.

In less preferred embodiments, the labels may be radioactive and a radioactivity detection device would then be required. Ideally such devices would be real-time radioactivity imagers. Less preferred are other devices relying on phosphor screens or autoradiography films for detection.

In embodiments of the method where template molecules are immobilised in containers (e.g. wells), sequencing reactions can be monitored for each container. This can be done using microplate readers to achieve high orders of parallelism. Such readers are available commercially for the measuring of fluorescence or radioactivity (e.g. Discovery™, FluoroCount™ or TopCount™ microplate readers from Packard Instrument Company).

Depending on the number of locations where detection of signals is desired, a scanning system may be preferred for data collection. (Although an alternative is to provide a plurality of detectors to enable all locations to be covered.) Such a system allows a detector to move relative to a plurality of locations to be analysed. This is useful when all the locations providing signals are not within the field of view of a detector. The detector may be maintained in a fixed position and locations to be analysed may be moved into the field of view of the detector (e.g. by means of a movable platform). Alternatively the locations may be maintained in fixed position and the detection device may be moved to bring them into its field of view.

The detection system is preferably operably linked to an analysis system in order to determine the number (and preferably also the nature) of bases incorporated by primer extension at each location after each step. This analysis may be performed immediately after each step or later on, using recorded data. The sequence of template molecules immobilised at a given location can then be deduced from the number and type of nucleotides added after each step.

Preferably the detection system is part of an apparatus comprising other components. The present invention includes an apparatus comprising a plurality of labelled nucleotides, a nucleic acid polymerase and detection means for detecting labelled nucleotides when incorporated into a nucleic acid molecule by primer extension, the detection means being adapted to distinguish between signals provided by labelled nucleotides incorporated at different locations.

The apparatus may also include temperature control, solvent delivery and washing means. It may be automated.

An apparatus as described above may be used in a highly preferred embodiment of the present invention. Here different template molecules can be sequenced as follows:

1) each type of DNA molecule is arrayed and covalently bound at a different location (e.g. a spot) of a flat solid surface;

2) the molecules are denatured to produce single-stranded DNA;

3) oligonucleotide primers are specifically annealed to the single-stranded DNA template molecules; unbound primers are removed;

4) a reagent mixture for nucleotide extension (containing e.g. a single type of deoxyribonucleotide, at least part of which are fluorescence labelled, a DNA polymerase and a suitable reaction buffer) is applied to the DNA array;

5) the DNA array is washed to remove unincorporated nucleotides;

6) the amount of fluorescence at each location is measured and recorded;

7) the reaction proceeds to step 4) using another type of nucleotide until a sufficient amount of sequence data has been generated.

Methods and apparatuses within the scope of the present invention can be used in the sequencing of:

- unidentified template molecules (i.e. *de novo* sequencing);
- and templates which are to be sequenced to check if one or more differences relative to a known sequence are present (e.g. identification of polymorphisms). This is sometimes referred to as "re-sequencing".

For *de novo* sequencing applications, the order of nucleotides applied to a given location can be chosen as desired. For example one may choose the sequential addition of nucleotides dATP, dTTP, dGTP, dCTP; dATP, dTTP, dGTP, dCTP; and so on. (Generally a single order of four nucleotides would be repeated, although this is not essential.)

The number of steps required for *de novo* sequencing a sequence of n bases is dependent on the template sequence: a high number of identical bases repeated in a contiguous stretch reduces the number of steps, whereas a template sequence having fewer such tandem repeats increases the number of steps. Without any identical base repeated contiguously, the maximum number of *de novo* sequencing steps required is $3n+1$. (After a given base has been incorporated, the next based to be added is one of the 3 other bases. The exception to this is the first step of primer extension where the first base is any of 4 bases). Thus, for templates without any repeated bases, the probable average number of steps required to sequence n bases is very close to $2n$ (considering that any of the 3 bases which may get incorporated at a given cycle has an equal probability to be added, thus meaning that in average a base will be added every 2 cycles). Thus, for biological templates which generally contain numerous bases in tandem, the actual number of cycles required for *de novo* sequencing will be lower than 2 times the number of bases to be sequenced. For a truly random sequence, it can be shown that the number of steps required to sequence n bases is, in average, $1.5n$ steps.

For re-sequencing applications, the order of nucleotides to be added at each step is preferably chosen according to a known sequence.

Re-sequencing may be of particular interest for the analysis of a large number of similar template molecules in order to detect and identify sequence differences (*e.g.* for the analysis of recombinant plasmids in candidate clones after site directed mutagenesis or for polymorphism screening in a population). Differences from a given sequence can be detected by the lack of incorporation of one or more nucleotides present in the given sequence at particular stages of primer extension. In contrast to most commonly used techniques, the present method allows for detection of any type of mutation such as point mutations, insertions or deletions. Furthermore, not only known existing mutations, but also previously unidentified mutations can be characterised by the provision of sequence information.

In some embodiments of the method, long template molecules may have to be re-sequenced by several sequencing reactions, each one allowing for determination of part of the complete sequence. These reactions may be carried out at different locations (e.g. each location with the same template but with a different primer), or in successive cycles (e.g. between each cycles the primers and extension products are washed off and replaced by a different primer).

The present invention will now be described by way of example only, with reference to the accompanying drawings. Unless the context indicates otherwise, techniques described with regard to DNA molecules should also be considered to be applicable to RNA molecules.

FIGURE 1 illustrates in schematic form the *in situ* sequencing of two different DNA molecules, each molecule being present in multiple copies at particular locations, as indicated by squares or circles.

FIGURE 2 illustrates how fluorescence readings can be used to determine a DNA sequence *in situ*.

FIGURE 3, 4, 5 and 6 illustrate apparatuses of the present invention.

FIGURES 7, 8 and 9 illustrate that *in situ* sequencing done via the present invention can be confirmed (if desired) by standard techniques using gels.

Figure 1

Referring now to Figure 1, individual spots or arrayed DNA templates are provided at different locations, each comprising large numbers of identical nucleic acid molecules.

Figure 1(a) shows the DNA templates before fluorescence labelled nucleotides are added.

Figure 1(b) shows the DNA templates after a fluorescence labelled dGTP has been added in the presence of a DNA polymerase and the DNA templates have been washed to remove any labelled dGTP not used in primer extension. The dark DNA templates are those which have incorporated one or more labelled Gs.

Figures 1(c), (d) and (e) show how the procedure illustrated by Figure 1(b) has been repeated using labelled nucleotides dATP, dTTP and dCTP respectively (and washing after each cycle to remove nucleotides not used in primer extension).

A fluorescence detection means can be used to distinguish between incorporation of fluorescence labelled A, T, G or C. The latest base to be incorporated at a given location by primer extension is illustrated in Figures 1(b) to 1(e) by filled circles having different appearances for different bases (G is represented by dark circles, A by circles with oblique shading, T by grid-like shading and C by dots).

Figure 1(f) shows two partial sequences which have been determined by the method illustrated in Figures 1(a) to (e). The sequence shown in lane 1 is GAC, whereas that shown in lane 2 is ATC. For ease of reference, the DNA templates

shown in Figure 1(a) have been identified with numbering corresponding to the lane numbering shown in Figure 1(f). It can thus be seen that two different types of DNA template are present in Figure 1(a) (i.e. a given DNA molecule is present in DNA templates identified with "1" in substantially homogenous form and with a different DNA molecule is present in DNA templates identified with "2", also substantially homogenous in form).

Of course the present invention can be used to sequence many more than two different nucleic acid molecule sequences and can be used for sequencing RNA as well as DNA.

Figure 2

Turning now to figure 2, *in situ* sequencing is illustrated using a particular primer annealed to a template.

Figure 2(a) shows the primer annealed to the template. The primer can be seen to have a 3' end available for primer extension.

Figure 2(b) shows how fluorescence-labelled bases complementary to the underlined bases shown in Figure 2(a) can be incorporated by a stepwise primer extension cycle using fluorescence-labelled nucleotides.

Figure 2(c) shows how bases with fluorescent labels, which can be incorporated by primer extension, can be detected *in situ* by fluorescence measurements.

Figure 3

Turning now to Figure 3, an apparatus is illustrated for performing the present invention.

The features shown are listed below:

1. Arrayed DNA samples and transparent cover plate.
2. Seal around cover plate.
3. Bottom plate.
4. Inlet for reagents. (A plurality of conduits could be provided.)
5. Electronically controlled valves.
6. Reservoir of reagents.
7. Detection device (e.g. CCD camera).
8. Outlet for reagents.
9. Electronically controlled valves.
10. Reservoir of reagents. N.B. The reagents can be recycled directly into reservoir 6.

In use, template molecules are arrayed onto a plate and are covered by a transparent cover (*e.g.* a quartz plate and a cover slip may be provided). The sides of the plate and cover are sealed so that liquid can be circulated between them. The reagents, which can be recycled, are distributed via a system of conduits and valves controlled electronically. Ideally a computer is used to program the sequential incubation of the samples with appropriate reagents under appropriate conditions. The same or another computer may also control

the detection system (*e.g.* a CCD camera and possibly means for moving the camera relative to the template molecules) and perform data analysis.

Figure 4

Figure 4 illustrates another apparatus for performing the present invention. This apparatus is preferred for manually controlled stepwise sequencing. The features illustrated are listed below:

1. Arrayed DNA samples.
2. Deformable absorbent material containing reagents, including appropriate type of nucleotide, to be used in primer extension.
3. Washing device.

In use, the deformable absorbent material (*e.g.* agarose gel or cellulose fibres) holds sequencing reagents (*e.g.* polymerase, buffer, and one type of nucleotide at a time). A soft pressure of the deformable absorbent material onto the surface where the template molecules are arrayed can liberate enough reagents for a primer extension reaction to occur, with minimal reagent waste. A washing step may then be performed by causing liquid to flow over the arrayed DNA samples, using the washing device.

Figure 5

An automated system, as shown in Figure 5, can also be designed which uses the deformable absorbent material and reagents held thereon. Ideally, four types of reagent mixes (one for each type of nucleotide) are provided by separate

areas of the deformable absorbent material. The deformable absorbent material may be generally cylindrical in shape and may have four different regions provided on the outer surface of a cylinder. The template molecules may be arrayed on the surface of another cylinder. The template cylinder may be of smaller diameter (*e.g.* $dt = 1/4dr$ where dt is the diameter of the template cylinder and dr is the diameter of the reagents cylinder) or larger diameter (e.g. $dt = dr + 1/4$ or $dt = dr + 3/4$). A continuous cycling of reactions can be achieved by rolling the reagent and sample cylinders against each other. Washing and detection can also be performed continuously along the surface of the template cylinder.

The automated system illustrated by Figure 5 will now be described in greater detail:

(Figure 5A shows a perspective view of the system. Figure 5B shows a plan view of the system.)

The components are as follows:

1.: Cylinder covered with deformable absorbent material containing sequencing reagents. Shown here is an example of cylinder with 4 sectors, which may each contain a different reagent (e.g. polymerisation mixtures, each one with a different nucleotide).

2.: Cylinder of smaller diameter (e.g. one fourth of the diameter of cylinder 1, as shown here) on which the samples are arrayed.

3.: Washing device.

4.: Detection device (e.g. CCD camera).

Typically, both cylinders roll against each other, so that the reagents on cylinder 1 are applied on surface of cylinder 2. The order of reagents application would correspond to their disposition on cylinder 1.

It is possible to modify this order of reagent application in other embodiments of the invention (e.g. for re-sequencing). For example, an additional device can be used to skip application of one or more reagents onto cylinder 2. This can be achieved by controlled rotation of cylinder 1 relative to cylinder 2 while contact between both cylinders is avoided.

<u>Figure 6</u>

A further apparatus, as shown in Figure 6, can be designed. Reagents can be dispensed by microspraying. Thus minimal volumes of reagents need be used at each step. For instance, automated piezoelectric devices can be designed to dispense nanolitres (or picolitres) of reagents on defined locations (e.g. ink-jet printing technology). The re-sequencing of different templates, or of similar templates from different primers, can be achieved by dispensing the appropriate reagents (*e.g.* primer in annealing solution or polymerase, buffer and nucleotide) at defined locations.

Three systems that can be used successively to carry out a sequencing reaction are illustrated in Figure 6:

A. Piezoelectric device for dispensing the appropriate reagents at defined locations. The device is linked to an electronic controller and to a reservoir of reagents.

B. Washing device.

C. Detection device.

EXAMPLES

Example 1

This example provides an indication that successful step-by-step incorporation of nucleotides using the method of the present invention can be achieved. The example uses a sequencing gel for illustrative purposes (see Figure 7) since such gels are routinely used for sequencing. It will however be appreciated that the present invention is preferably used for *in situ* sequencing of nucleic acid molecules and therefore it does not require the use of sequencing gels.

Experimental procedure:

1. The sequencing cycles were done on single-stranded template molecules bound on magnetic beads.

The 174 base pair DNA fragment was amplified by PCR from the multiple cloning site of plasmid pBlueScript SK- (Stratagene). The forward primer (5'-biotin-GCGCGTAATACGACTCACTA-3') and reverse primer (5'-CGCAATTAACCCTCACTAAA-3') were located on position 621 and 794, respectively. The amplified double-stranded DNA was bound on streptavidin coated magnetic beads (Dynabeads M-280, Dynal, Oslo) and denatured with NaOH. After washing, the single-stranded DNA bound to the beads was kept in 10 mM tris pH 8.0.

2. The reverse primer was used for sequencing. It was labelled on its 5' end with radioactive phosphate, and annealed to the single-stranded DNA template. The primer molecule is expected to be extended with the following bases : GGGAACAAAAGCTGGAG...

The primer end-labelling was done with polynucleotide kinase (Pharmacia) in presence of [γ-^{32}P] ATP as described by manufacturer. After purification on Sephadex G-25 spin column, the primer were conserved at -20°C.

The annealing was performed by heating the mixture of primer and template molecules in 1x Sequenase buffer [40 mM Tris pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl] for 5 min at 70°C and slow cooling for 2 hours.

3. The sequencing reaction were performed by successive cycles of primer extension (in presence of only one type of nucleoside triphosphate), aliquot removal for analysis and washing. Each cycle was done using a different deoxyribonucleoside triphosphate in solution, using the arbitrary order of dATP, dTTP, dGTP, dCTP, dATP, dTTP, dGTP, dCTP, dATP, dTTP, etc.

We choose the T7 Sequenase II (Amersham) as the enzyme for polymerisation reactions. In contrast to recommendations of the manufacturer the reaction mix only contained 0.1 u/µl Sequenase and 100 nM of a single type of normal deoxyribonucleoside triphosphate in 1x Sequenase buffer. Four different mixes were prepared, each with a different type of nucleoside triphosphate.

A cycle started with the removal of buffer from the template-bound beads using a magnet. Ice-cold reaction mixture was added. After 5 seconds an aliquot was collected and kept on ice in 10 volumes of stop buffer containing 90% formamide and 20 mM EDTA. Ten volumes of washing buffer (10 mM Tris pH 8.0) were added to the remaining reaction mixture and immediately removed after magnetic separation of the template-bound beads. Washing was repeated and next cycle was initiated.

At each cycle, a smaller volume of fresh reaction mixture was used, according to the remaining amount of template after aliquot removal and washing losses.

4. The aliquots collected after each cycle were then analysed on denaturing sequencing gel. After migration, the gel was autoradiographed to reveal to position of each band.

The aliquots in stop buffer were denatured for 5 min at 95°C and immediately cooled on ice before loading on 0.75 x 180 x 320 mm 15% acrylamide gels containing 1xTBE buffer and 7 M urea. The system was model SE420 from Hoefer Scientific Instruments. Migration was at 2000 V for 5 min and 250 V overnight.

As a control, unreacted oligonucleotides were applied on gel (lane 1), as well as the product of a reaction performed in presence of all 4

nucleosides triphosphate, to give rise to fully extended molecules (lane 2).

After migration, the gel was fixed in 10% glacial acetic acid and 10% methanol for 30 min. Still humid, it was transferred in a plastic bag in autoradiography cassette to expose a Kodak X-OMAT AR film for 9h at 25°C.

The very high sensitivity of radio-active label, associated with gel analysis of extended fragments after each cycle allowed careful monitoring of the reaction. As shown on Figure 7 it was demonstrated that:

- Conditions were identified which permit correct polymerisation in the presence of only one type of nucleoside triphosphate. The polymerisation proceeds completely to the last base to be incorporated (lane 5), but is blocked for (further) extension if the complementary base is not present on template molecule (lane 3, 4, or 5).

- There is no misincorporation, as seen on lanes 3, 4, or 6.

- There is no 3'-5' exonuclease activity (e.g. fragments of shorter size are not detected on lane 3 or 4).

- Successive cycles can be carried out correctly.

Only fragments of the correct size are observed. There are three exceptions : (1) a small proportion of the primer molecules were not extended during first cycle, probably because of 3' end damage of the oligonucleotide. (2) A proportion of full length molecules are observed after cycle 13, which suggests that washing was not complete and that the 4 nucleosides triphosphate were present in solution. This problem is not considered as relevant, because it was not observed between cycles 4 and 12, even though the 4 different nucleosides triphosphate had already been used for reactions. The washing steps should also be facilitated when the experiment will be repeated with template molecules bound on plastic surface. (3) Faint bands of smaller size than expected could be detected after longer exposure, which suggests uncompleted reaction. However, the proportion is so low that it should not interfere with *in situ* detection.

Only 19 cycles were carried out in this preliminary experiment, but there is no reason why more cycles (up to 50 or up to 100 or more) could not be performed successfully.

This experiment demonstrated that not only re-sequencing (when only the expected nucleoside triphosphate is used for polymerisation), but that also *de novo* sequencing can be carried out efficiently with this method.

After 20 cycles of *de novo* sequencing of an unknown template molecule, the minimal number of bases which could be sequenced is of 6, the maximal number depending on the base order and on the presence of runs of the same

base. In this *de novo* sequencing experiment, as many as 17 bases were read in 19 cycles.

It should also be mentioned that the template molecule used here is considered as difficult to sequence because of its high content of palindromic regions, which gives rise to secondary structures in single-stranded DNA. This potential difficulty appeared not to be a problem in our experiment.

Example 2: Re-sequencing using non-labelled primers

This example provides a further indication that successful step-by-step incorporation of labelled nucleosides triphosphate can be achieved using the method of the invention. Like in Example 1, a sequencing gel was used for illustrative purposes (Figure 8). It will however be appreciated that the present invention is preferably used for *in situ* sequencing of nucleic acid molecules and therefore it does not require the use of sequencing gels.

Template preparation:

A 608 base pair DNA fragment inserted into the polylinker of pBlueScript SK^- plasmid was amplified by PCR. The forward primer (5'-GCG CGT AAT ACG ACT CAC TA-3') was biotinylated on its 5'-end and the reverse primer (5'-GCA ATT AAC CCT CAC TAA A-3') was not functionalised (i.e. -OH). The amplified double-stranded DNA was purified on a spin column (Pharmacia MicroSpin S-400 HR) to remove unused primers. A hundred microlitres of purified DNA were then diluted in a total volume of 400 µl and bound on same volume of streptavidin coated magnetic beads (10 mg/ml) according to the manufacturer's protocol (Dynabeads M-280, Dynal, Oslo). The DNA was denatured in presence of 0.1 N NaOH and beads were washed to remove the complementary strand. The single-stranded DNA bound to the beads was resuspended in 100 µl 10 mM tris pH 8.0 and conserved at 4°C.

Primer annealing:

The primer 5'-TAC CAG TTT CCA TTC CAG C-3' was used for sequencing. The annealing was performed by heating the primer and template mixture for 5 min at 95°C and cooling to 60°C in 30 min. Typically, 20 μl of beads were annealed with 10 pmol of primer in 100 μl of 1x Sequenase buffer (40 mM Tris pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl). The primer was expected to be extended with the following bases: CGCTGGGGTGGTTT... which are complementary to the template sequence.

The primer extension reactions were performed in presence of only one type of nucleoside triphosphate. Each step was done using a different deoxyribonucleoside triphosphate in solution, using the expected order of dCTP, dATP, dTTP, dGTP, dCTP, dTTP, dGTP, dTTP, dGTP, dTTP, etc..(the first dATP and dTTP will not get incorporated and serve to demonstrate absence of misincorporation). The T7 DNA polymerase Sequenase 2.0 (Amersham) was chosen for polymerisation reactions. The reaction mix contained 0.2 u/μl Sequenase, 250 nM of a single type of normal deoxyribonucleotide and 5 nM of the same type of [α-^{32}P]-labelled deoxyribonucleotide (50 nCi/μl, Amersham) in 1x Sequenase buffer. Four different mixes were prepared, each with a different type of nucleoside triphosphate.

The first polymerisation step was initiated by sedimenting 20 μl of template-bound beads. The beads were resuspended in 25 μl of ice-cold reaction mix and reaction was allowed to proceed for 10 seconds.

The beads were immediately sedimented with the magnet and washed 3 times for 1 min in 10 volumes of 1x Sequenase buffer. After the final wash, the beads were resuspended in the initial volume of 1x Sequenase buffer. One microlitre aliquot was collected after each cycles and kept in 10 µl stop buffer (90% formamide, 20 mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol FF). The next polymerisation and washing steps were performed as described *supra* using adequate volumes of reagents (i.e. each step using smaller volumes to compensate for aliquot removal).

After final washing step, sample were analysed by gel electrophoresis. The aliquots in stop buffer were denatured for 5 min at 95°C and immediately cooled on ice before loading on 0.75 x 180 x 320 mm 15% acrylamide gel containing 1x TBE buffer and 7 M urea. The electrophoresis system was model SE400 from Hoefer Scientific Instruments. Migration was at 2000 V for 5 min and 250 V overnight. After migration, the gel was immediately transferred into a plastic bag to expose a Kodak X-OMAT AR film in an autoradiography cassette for 3 to 16h at 25°C.

As discussed *supra* and shown in Figure 8, this example indicates that the sequencing method of the invention can be successfully adapted for stepwise sequencing from non-labelled primers.

Example 3: Sequencing template bound in a microplate well

Template preparation:

In this example, DNA molecules of approximately 700 bases were used as template. They were bound on the plastic surface of a modified microplate as described by the manufacturer (NucleoLink™ from NUNC A/S, Roskilde, Denmark).

Two types of DNA templates (named A and B) were bound to the wells, either individually or mixed (50% each).

Primer annealing:

A 20 base-long oligonucleotide was used as sequencing primer (5'-GGT CAG GCT GGT CTC GAA CT-3') specific for DNA template B. The annealing was performed by heating the microplate coated with template for 4 min at 94°C and slow cooling to 60°C in 30 min, in presence of 100 nM primer (in 20 μl 5x SSC buffer + 0.1% Tween® 20 per well). The primer was expected to be extended with the following bases: CCCTACCTCA... which are complementary to the template sequence.

Polymerisation step:

The primer extension reactions were performed in presence of only one type of nucleoside triphosphate. Each step was done using a different nucleoside triphosphate in solution, using the expected order of dCTP, dTTP, dATP, dCTP, dTTP, dCTP, dATP. The Klenow fragment of DNA polymerase I (3'->5' exo$^-$, New England Biolabs) was used for polymerisation reactions. The reaction mix

contained 0.25 u/μl Klenow exo$^-$, 50 nM of a single type of normal deoxyribonucleoside triphosphate and 20 nM of the same type of [α-^{32}P]-labelled deoxyribonucleoside triphosphate (50 nCi/μl, Amersham) in 1x Polymerase I buffer (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol). Four different mixes were prepared, each with a different type of nucleoside triphosphate. Typical reactions were performed in 12 μl.

A polymerisation step was initiated with the removal of reagents from the well. The adequate volume of reaction mixture was added and reaction was allowed to proceed for 30 seconds at room temperature.

Washing step:
The wells were immediately washed 3 times for 1 min in 100 μl of TNT buffer (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween® 20).

Detection step:
After each washing step, the radioactivity incorporated on each well was measured and recorded using a scintillation counter.

Sequencing cycle:
In this example, the polymerisation, washing and detection steps were be repeated sequentially.

Results shown in Figure 9 indicate that primer extension occurred as expected. The stepwise counts increase occurred specifically in presence of template B, and was dependent on its amount bound on wells (e.g. approximately twice as

much counts in presence of 100% template B than in presence of 50% template B). After each step, the number of counts detected increased almost proportionally to the number of bases added.

Example 4: Sequencing using fluorescent label

The experiment described in example 3 may be repeated with radioactive labels being replaced by fluorescent ones. Fluorescein-labelled nucleoside triphosphates (Fluorescein-12-dNTP, DuPont) may be used. In such an experiment, nucleotide incorporation could be detected using a microplate fluorescence reader.

Use of double stranded, nicked molecules

Although the foregoing examples have been generally based upon a method in which primers are hybridised to a single-stranded nucleic acid molecule and the primers are then extended, it is possible to use an alternative approach in which a double stranded molecule having a nick is provided (a nick being a gap in one strand of a double-stranded DNA molecule and allowing a free 3'-OH end to be provided).

The nick provides a 3'-OH group which acts as a starting point for chain extension in the presence of a suitable polymerase and a supply of nucleotides. The 3'-OH group provided via a nick therefore has an equivalent function to the 3'-OH group at the end of a primer in the methods discussed previously. The nick can be provided by any suitable means. For example it can be provided by using restriction enzymes on DNA molecules with hemiphosphorothioated recognition sites. (Reference : Spears, P.A., Linn, C.P., Woodard, D.L., Walker, G.T. (1997). Simultaneous strand displacement amplification and fluorescence polarization detection of *Chlamidia trachomatis* DNA. Analytical Biochemistry 247: 130-137).

Claims

1. A method for sequencing nucleic acid molecules, comprising the steps of:

a) providing at a first location a plurality of single stranded nucleic acid molecules that have the same sequences as one another and that are hybridised to primers in a manner to allow primer extension in the presence of nucleotides and a nucleic acid polymerase;

b) providing at a second location, which is different from the first location, a plurality of single stranded nucleic acid molecules that have the same sequences as one another, but that have different sequences from the sequences of the single stranded nucleic acid molecules at the first location, and that are also hybridised to primers in a manner to allow primer extension in the presence of nucleotides and a nucleic acid polymerase;

c) providing each location with a nucleic acid polymerase and a given labelled nucleotide under conditions that allow extension of the primers if a complementary base or if a plurality of such bases is present at the appropriate position in the single stranded nucleic acid molecules;

d) detecting whether or not said labelled nucleotide has been used for primer extension at each location by determining whether or not the label present on said nucleotide has been incorporated into extended primers;

e) repeating steps c) and d) one or more times so that extended primers comprising a plurality of labels are provided.

2. A method according to claim 1, wherein all or part of the sequence that is obtained in step e) is converted to provide a complementary sequence thereto.

3. A method according to any preceding claim, wherein if the given nucleotide has been used in primer extension in step d) then this step includes the step of detecting how many of the given nucleotides have been used per extended primer.

4. A method according to any preceding claim, wherein after step c) excess nucleotides that have not been used in primer extension are removed (e.g. by washing).

5. A method according to any preceding claim, wherein step d) uses absorption or emission spectrometry

6. A method according to any preceding claim, wherein said single stranded nucleic acid molecules, said primers or both of the aforesaid are immobilised.

7. A method according to any preceding claim that is used to fully or partially sequence 10 or more nucleic acid molecules having different sequences simultaneously.

8. A method according to any preceding claim that is used to fully or partially sequence 100 or more nucleic acid molecules having different sequences simultaneously.

9. A method according to any preceding claim that is used to fully or partially sequence 1000 or more nucleic acid molecules having different sequences simultaneously.

10. A method according to any preceding claim, wherein each of four different nucleotides is used in primer extension.

11. A method according to claim 10, wherein said four different nucleotides are used in a predetermined order in repeated cycles.

12. A method according to claim 10 or claim 11, wherein the nucleotides are dATP, dTTP, dGTP, dCTP in labelled form.

13. A method according to claim 10 or claim 11, wherein the nucleotides are ATP, UTP, GTP, CTP in labelled form.

14. A method according to any preceding claim, wherein the detection step is carried out without moving the nucleic acid molecules from the different locations.

15. A method as described in any preceding claim with the exception that double stranded nucleic acid molecules having nicks therein are provided at the

first and/or second locations instead of providing single stranded molecules hybridised to primers.

16. A method as described in any preceding claim with the exception that only one nucleic acid molecule is provided at the first and/or second locations.

17. An apparatus for performing a method according to any preceding claim, the apparatus comprising a plurality of nucleotides, a nucleic acid polymerase and detection means for performing step d) of claim 1 or an equivalent step for claim 15 or claim 16, the detection means being adapted to distinguish between said different locations.

18. An apparatus according to claim 17 comprising means for removing excess nucleotides from the first and second locations (e.g. washing means).

19. An apparatus according to claim 18 or claim 19 that is automated to allow repeated cycles of primer extension and detection.

20. The invention substantially as hereinbefore described.

ABSTRACT

INVENTION

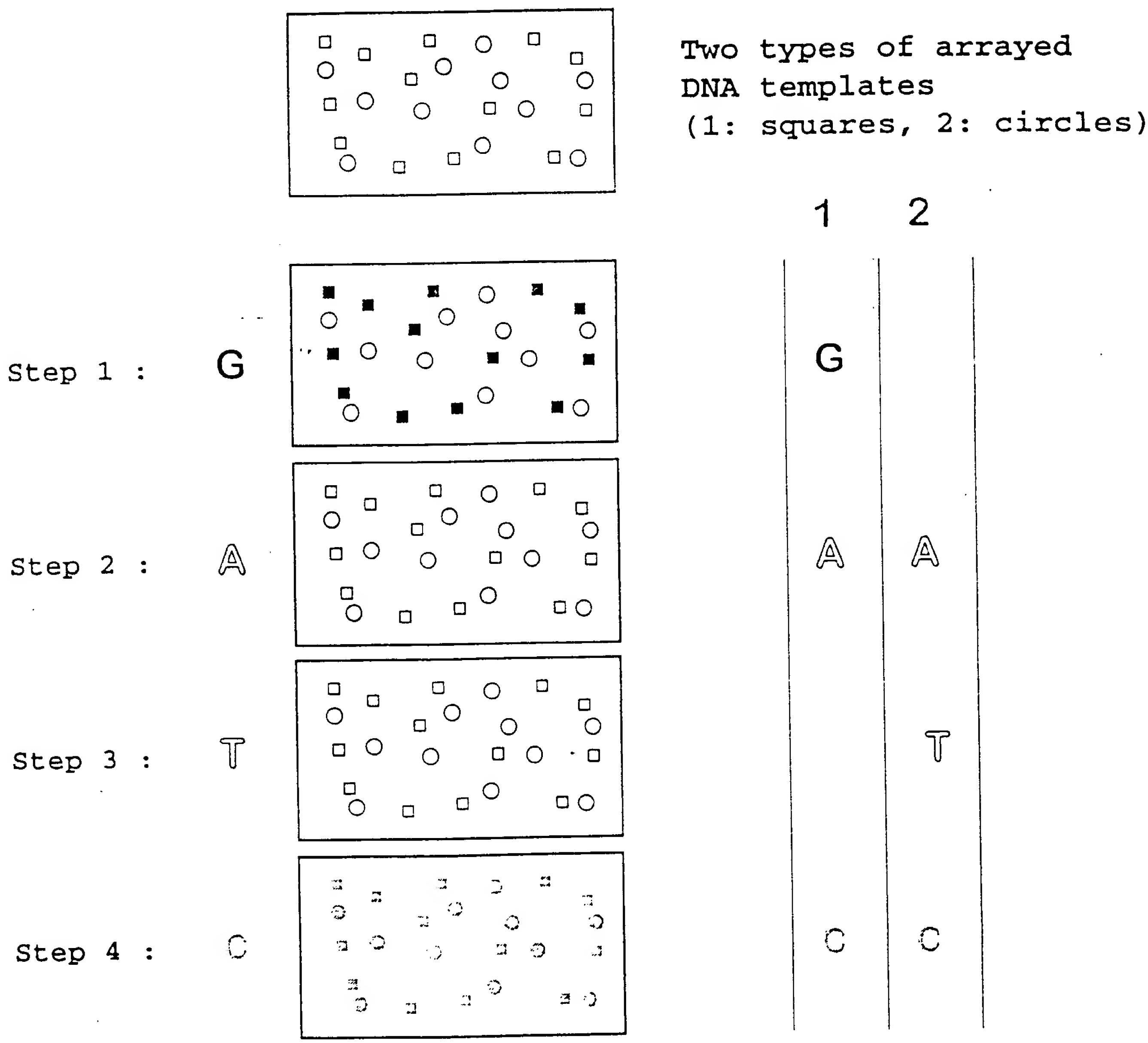
Different nucleic acid molecules present at different locations can be sequenced in parallel.

Primers that are annealed to the nucleic acid molecules can be provided. Each location can then be provided with a nucleic acid polymerase and a nucleotide. It can then determined whether or not the nucleotide has been used in primer extension and the process can be provided.

As an alternative to using primers, a nick in a double stranded nucleic acid molecule can provide a 3'-OH group for chain extension.

Figure 1

in situ Sequencing



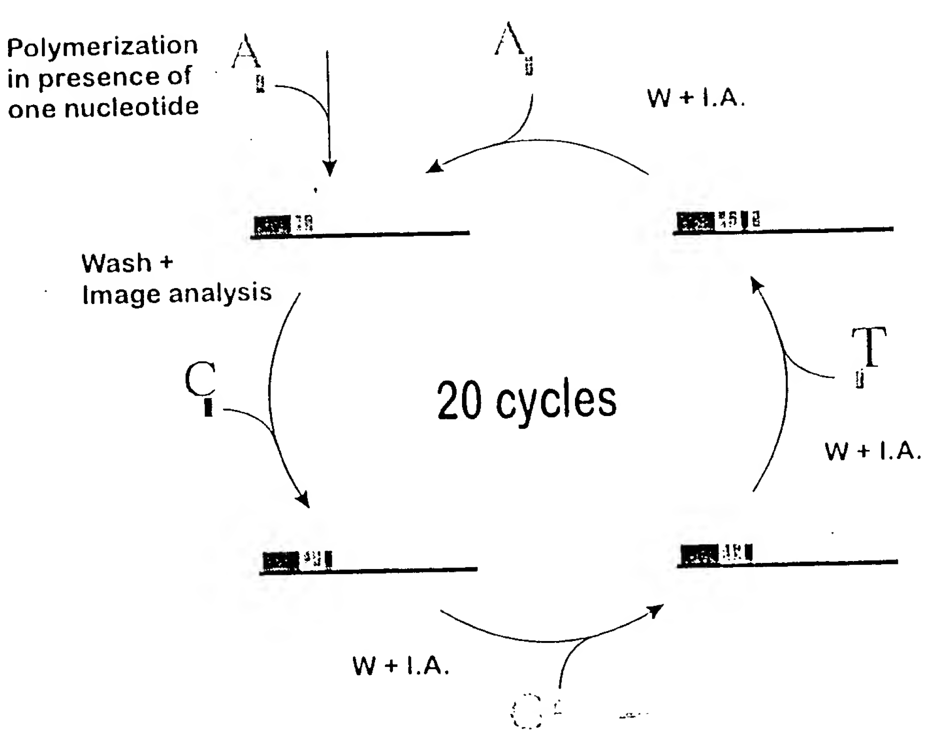
Sequence from type 1 : G A C
type 2 : A T C

Figure 2

in situ Sequencing

```
Primer :          5'-gactagcgtcat-3'
                     ||||||||||||
Template :   3'-ggatgctgatcgcactattgatgggcacgaactca-5'
```

Cycle of stepwise base extension



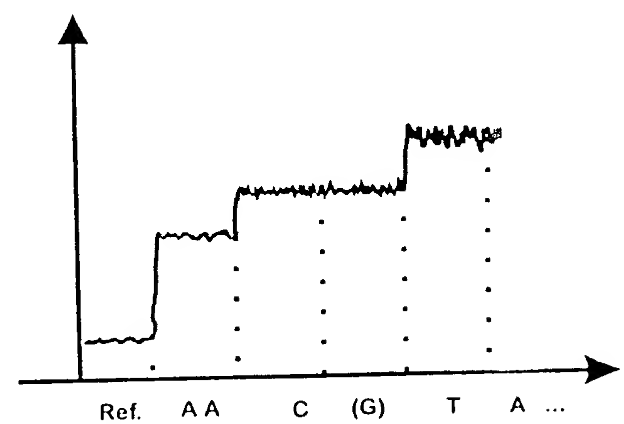


Figure 3

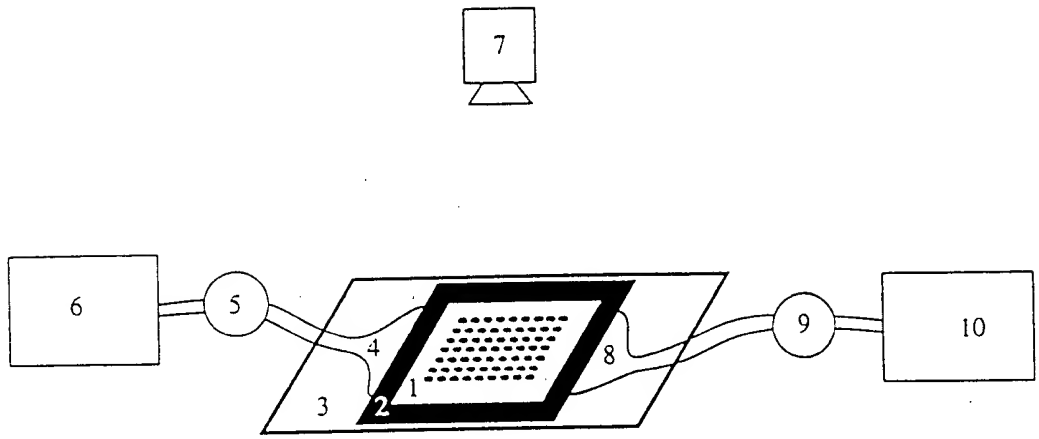


Figure 4

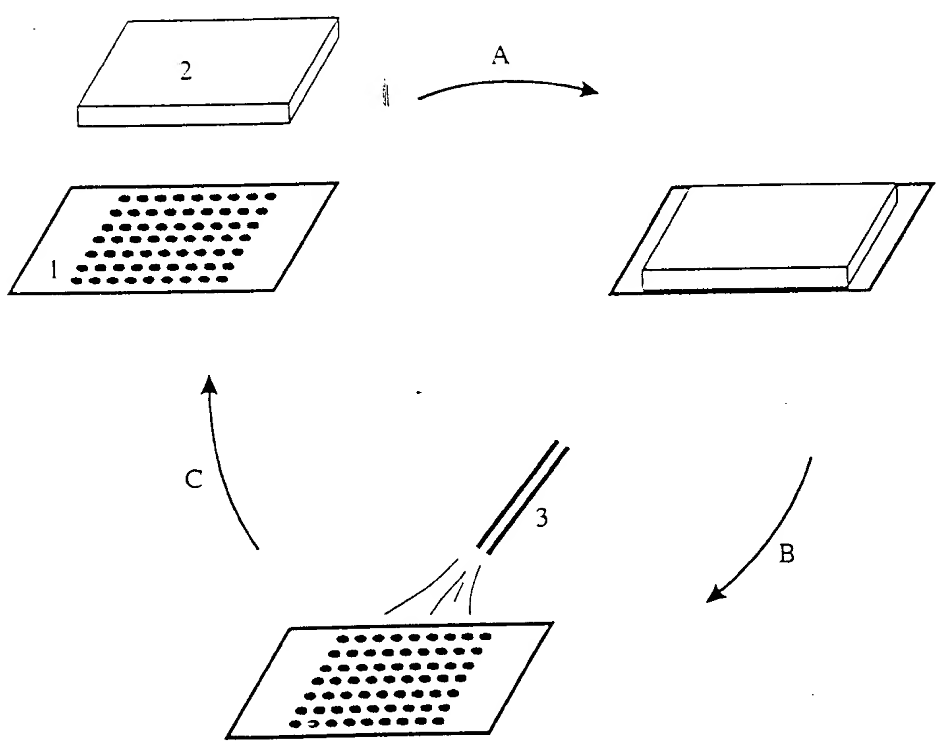


Figure 5

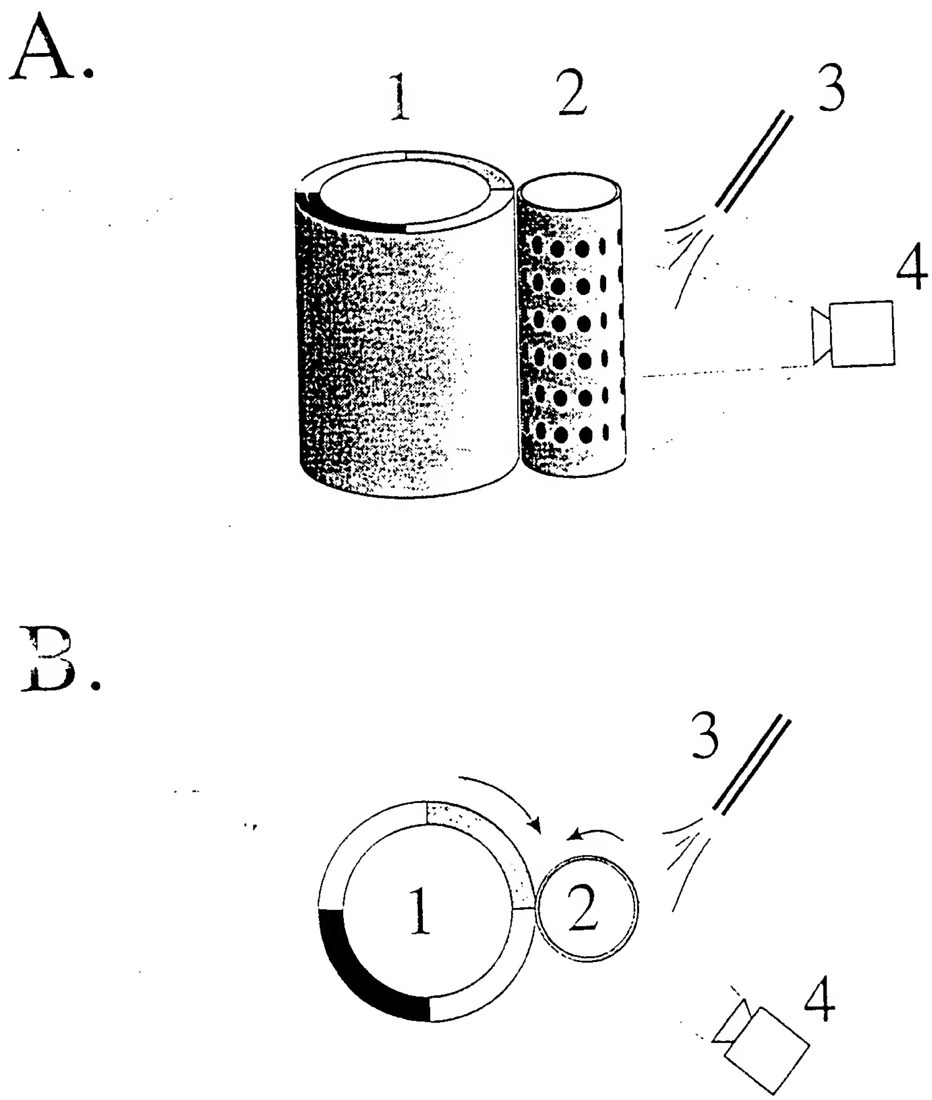


Figure 6

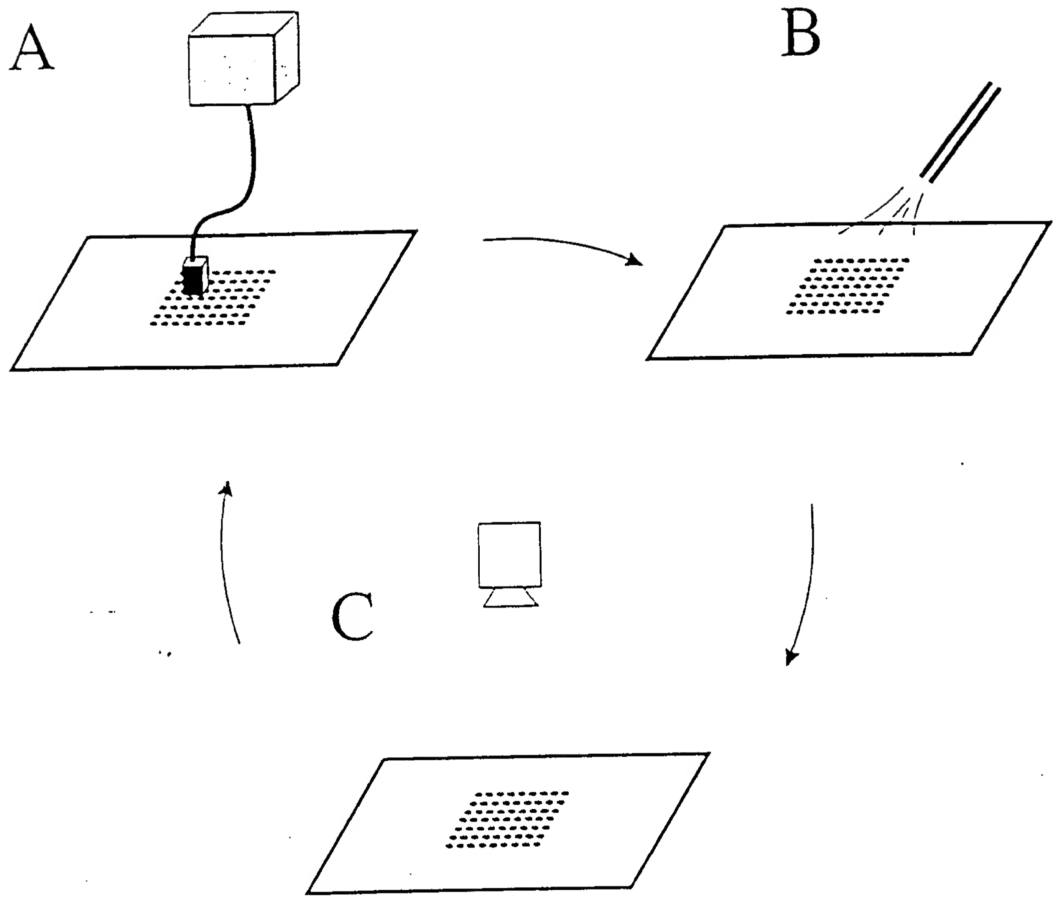
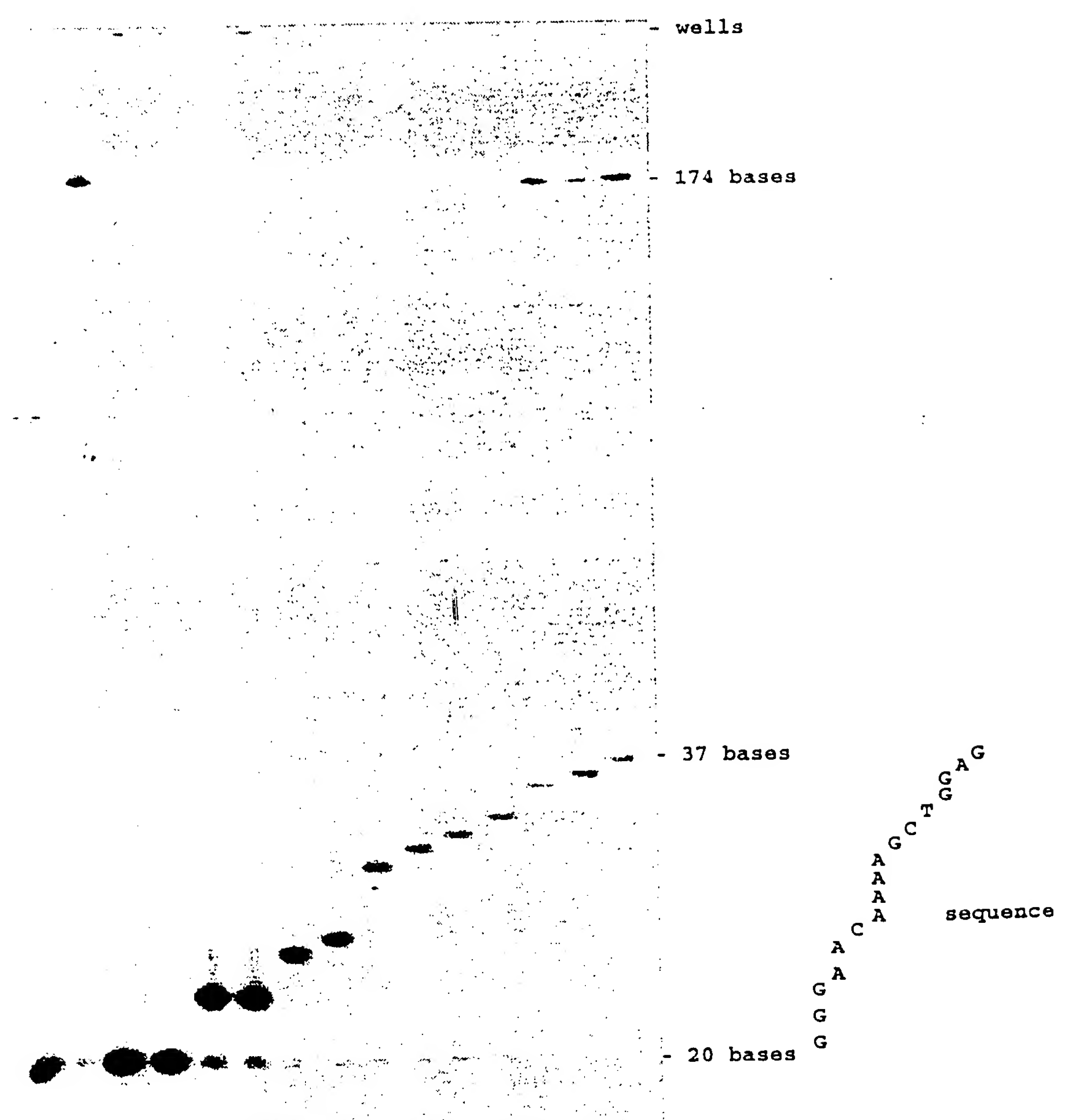


Figure 7

in situ Sequencing : *de novo* Sequencing

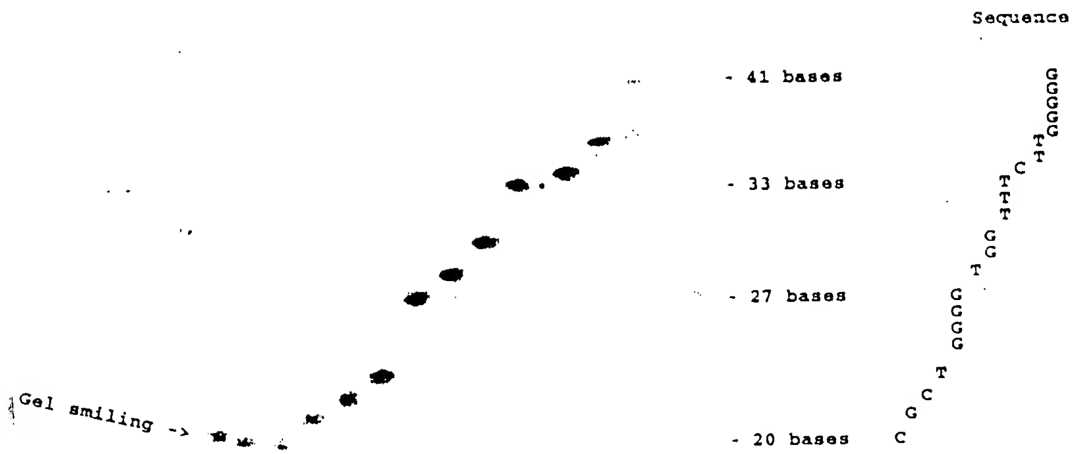


Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Nucleotide added	-	N	A	T	G	C	A	T G C	A	T G	C	A T	G	C A	T G
Cycle		1	1	2	3	4	5	6 7 8	9	10 11	12	13 14	15	16 17	18 19

0.75 mm 15% Acrylamide gel, TBE + 7M Urea

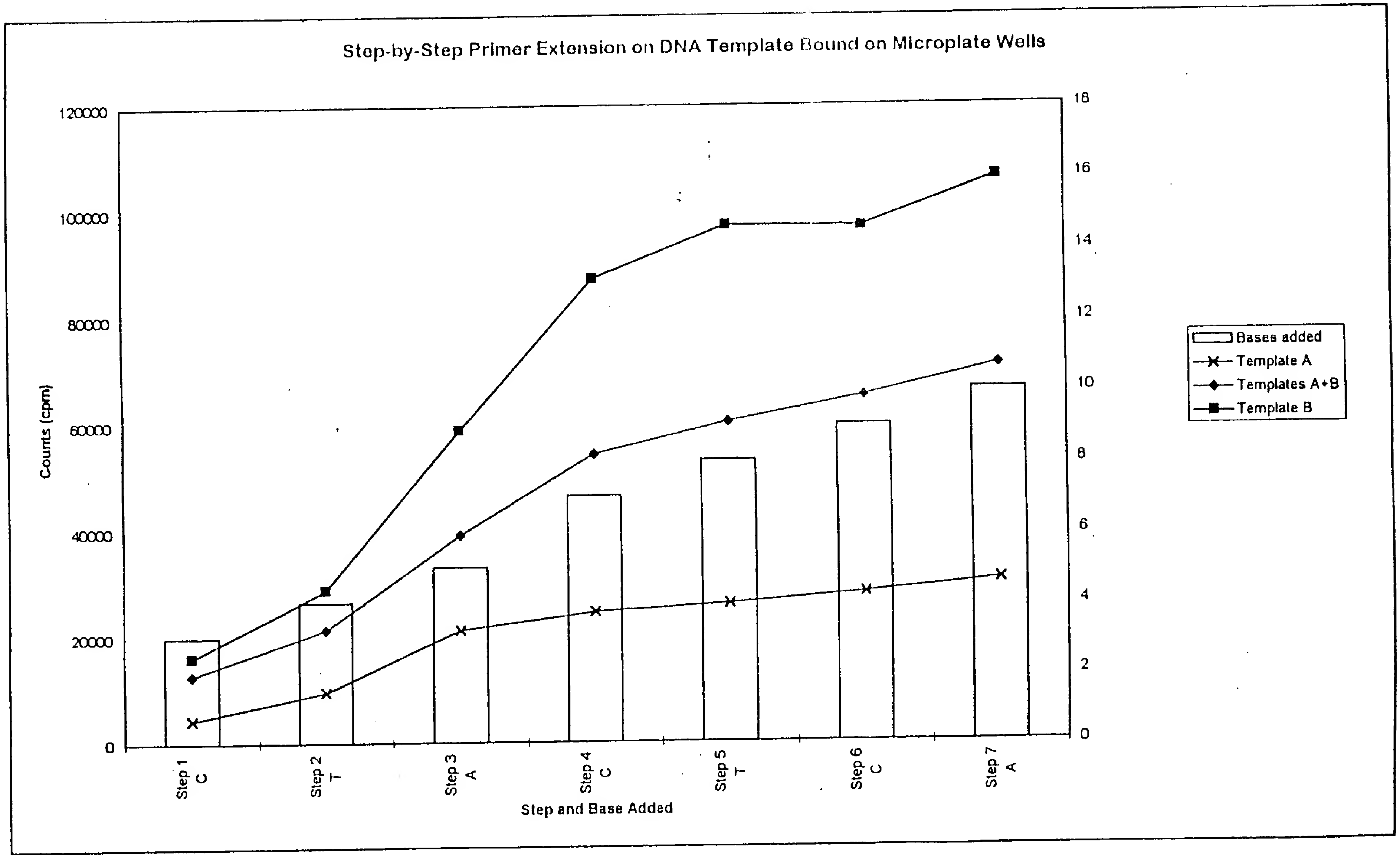
Figure 8

in situ Sequencing : Non-labelled primers



Lane / Step	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Nucleotide added	C	A	T	G	C	T	G	T	G	T	C	T	G	C	N
Number of based added	1	0	0	1	1	1	4	1	2	3	1	2	5	5	n

Figure 9



Step-by-Step Primer Extension on DNA Template Bound on Microplate Wells
Counts (cpm)
120000
100000
80000
60000
40000
20000
0
18
16
14
12
10
8
6
4
2
0
Step 1 C
Step 2 T
Step 3 A
Step 4 C
Step 5 T
Step 6 C
Step 7 A
Step and Base Added
Bases added
Template A
Templates A+B
Template B

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